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Changes in respiratory and photochemical activities during the conjugation process of *Spirogyra*

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Significant changes in activities of several enzymes were observed during the conjugation process of *Spirogyra*. The activity of endogenous respiration of intact cells increased temporarily during the early stages of conjugation, decreased sharply in the later stages, and reincreased very slightly at the new zygote stage. The activity of photo-oxidation of indigocarmine decreased gradually during the process. The following enzyme activities in the cell-free enzyme preparations decreased significantly during the process: NADH-oxidase, cytochrome *c* oxidase, NADH-cytochrome *c* reductase, succinate-cytochrome *c* reductase (mitochondrial fraction), NADP-photoreductase, cytochrome *c* photo-oxidase, cytochrome *c* photoreductase (chloroplast fraction), and triosephosphate dehydrogenase (soluble fraction). The amounts of chlorophyll *a* and *b* in the intact cells also decreased during the process.

For several years, we have been concerned with the physiological and biochemical studies on the conjugation phenomenon of *Spirogyra* (SASAKI *et al.*, 1966; YAMASHITA *et al.*, 1968; SASAKI *et al.*, 1972; SASAKI and TAKAYA, 1972). This alga is interesting material for studying the relationship between metabolic and morphological changes in the life cycle of plants because the cell size is comparatively large, a specific change in the cell form occurs synchronously during the conjugation process, and zygote is synchronously germinated by illumination of red light after the shorter dormant period.

In this paper, changes in the respiratory and photochemical activities of *Spirogyra* during the conjugation process are reported.

Materials and Methods

Plant materials: Two types of *Spirogyra*, Type I (cell width, approx. 70 μm) and Type II (cell width, approx. 115 μm) were used throughout this study. Multicellular algae in chain form were harvested at 10 o'clock each day at different stages of life cycle from the pond on the campus of Hokkaido University, washed well with sterile deionized water, and used as fresh intact cells. In the experiments with intact cells, the materials were cutted to 10 mm of length and soaked into 10 mM phosphate buffer, pH 7.2, and the enzymatic activities were assayed at 11 o'clock each day. In the experiments

with cell-free preparations, the materials were quickly frozen at −20°C and stored until use. The life cycle was divided into vegetatively growing (V-cell), conjugating (T-cell), and zygote (Z-cell) stages. The conjugation stage was further divided into pairing (P-cell), conjugation tube-forming (T_1-cell), conjugation tube-growing (T_2-cell), conjugation tube-connecting (T_3-cell), and protoplasm-translocating (T_4-cell) stages.

Preparation of cell-free enzyme: 1) For mitochondrial preparation, the frozen materials were homogenized in a Potter-Elvehjem glass homogenizer by addition of two volumes of medium consisted of 250 mM sucrose, 20 mM phosphate buffer (pH 7.2), and 0.2 mM versen; and 2) for chloroplast preparation, homogenizing medium consisted of 350 mM NaCl, 20 mM phosphate buffer (pH 7.2) and 0.2 mM versen was used (NIEMAN and VENESSLAND, 1959). The homogenate was filtered through three layers of cheese-cloth, and centrifuged at $500 \times g$ for 1 to 2 min. The supernatant was dialyzed against same medium for 3 hr or overnight in a cold room, and used as "crude cell-free extract". The extract was further centrifuged successively at $2{,}000 \times g$, $15{,}000 \times g$, and $25{,}000 \times g$ for 30 min. Each pellet was washed with the same medium twice, and was used as "chloroplast fraction", "mitochondrial fraction", and "small particulate fraction", respectively. The supernatant at $25{,}000 \times g$ was dialyzed against the same medium and was named as "soluble fraction". All above procedures were performed below 4°C.

Enzyme assays with intact cells: Endogenous respiratory activity was manometrically assayed with intact cells (200 mg of fresh weight) in 2 m*l* of 20 mM phosphate buffer, pH 7.2, at 25° or 30°C for 60 min, and the activity was expressed as Q_{O_2}. In some cases, the activity was assayed with glucose or succinate. The activity of photo-oxidation of indigocarmine was measured in 5.0 m*l* of a reaction mixture consisted of 50 mg fresh weight of cells, 150 μmoles of phosphate buffer, pH 7.2, 0.3 μmole indigocarmine reduced by dithionite, and 15 μmoles of NaN_3, in evacuated Thumberg tube. The reaction was run at 20°C for 10 min in light, 10,000 Lux, and the rate of oxidation of the dye was measured colorimetrically at 750 nm. The activity was expressed as μmoles of dye oxidized per mg dry wt per 10 min.

Assays of enzymes in mitochondrial fraction: The activities of respiratory enzymes in mitochondrial fraction or crude extract (equivalent to 0.5 mg of protein) were assayed in 2.0 m*l* of the reaction mixture containing following components: 1) for NADH oxidase, 150 μmoles phosphate buffer (pH 7.2), and 30 nmoles NADH; 2) for NADH-cytochrome *c* reductase, 150 μmoles phosphate buffer (pH 7.2), 30 nmoles NADH, 30 nmoles cytochrome *c*, and 3 μmoles NaN_3; 3) for succinate-cytochrome *c* reductase, 150 μmoles phosphate

buffer (pH 7.2), 20 μmoles succinate, 30 nmoles cytochrome *c*, and 3 μmoles NaN_3; and 4) for cytochrome *c* oxidase, 150 μmoles phosphate buffer (pH 7.2), and 30 nmoles reduced cytochrome *c*. Reaction time was 2 min at 20°C in darkness. Absorption changes were measured spectrophotometrically using a Hitachi Perkin-Elmer (Type 139) spectrophotometer, and the activities were expressed as nmoles of oxidized electron donors (or reduced acceptors) per min per mg protein.

Assays of enzymes in chloroplast fraction: The photochemical activities in chloroplast fraction (equivalent to 0.5 mg protein) were assayed in 2.0 m*l* of the reaction mixtures containing following components: 1) for NADP-photoreduction, 150 μmoles phoshate buffer (pH 7.2), 30 nmoles NADP, 30 μmoles $MgSO_4$, and 3 μmoles NaN_3 (JACOBI and DERNER, 1962); 2) for cytochrome *c* photoreduction, 150 μmoles phosphate buffer (pH 7.2), 30 nmoles NADP, 30 nmoles cytochrome *c*, 30 μmoles $MgSO_4$ and 3 μmoles NaN_3; and 3) for cytochrome *c* photo-oxidation, 150 μmoles phosphate buffer (pH 7.2), and 30 nmoles reduced cytochrome *c* (TAKAMATSU *et al.*, 1959). All above reactions were allowed in light, 10,000 Lux, at 20°C for 2 min, and the activities were expressed as nmoles of oxidized electron donor (or reduced electron acceptor) per min per mg protein.

Assay of triosephosphate dehydrogenase in soluble fraction: The activity in soluble fraction (equivalent to 0.5 mg of protein) was assayed in 2.0 m*l* of a reaction mixture consisted of 150 μmoles phosphate buffer (pH 7.2), 1 μmoles NAD or NADP, 5 μmoles glyceraldehyde 3-phosphate, 15 μmoles reduced glutathione, 17 μmoles Na_2AsO_4, 10 μmoles KF, and 10 μmoles $MgSO_4$ (BLACK and HUMPHREYS, 1962). The amounts of NADH or NADPH formed at 20°C for 2 min in darkness was measured spectrophotometrically, and the activity was expressed as nmoles of NAD or NADP reduced per min per mg protein.

Chlorophyll contents: Contents of chlorophyll *a* and *b* of intact cells were determined by the method of ARNON *et al.* (1949).

Protein was determined by the method of LOWRY *et al.* (1951), with bovine serum albumin as the standard.

Cytochome *c* (horse heart, Type II), NAD, NADP, glyceraldehyde 3-phosphate, glutathione and sodium dithionite were purchased from Sigma Chemical Co., and indigocarmine from Wako Pure Chemical Industries, Ltd.

Results

Conjugation period and respiratory activity: A relationship between conjugation period and respiratory activity was examined for several years

(1959 to 1966). Type I conjugated in 1959 to 1962 and 1967 to 1972, and Type II in 1959 to 1966. As shown in Figs. 1 and 2, Type I conjugated from about the 10th to the 20th of June for four years (1959 to 1962), and Type II at about the 10th of July to August. Type I conjugated at almost same period which was named as "usual conjugation period", therefore the period is restricted only once in a year. Usually, Type II grew well after the termination of conjugation process of Type I, and then conjugated from the early part of July to August in the same pond (1959-1962). In the years in which Type I could not grow well and did not conjugate, growth of Type II began from the early part of May and conjugated from the early part of June to August several times in a year (1963, 1964). In the years in which Type I conjugated at the usual conjugation period, fine weather continued prior to the initiation of the conjugation process. The length of time taken from the initiation to termination of the conjugation process was shorter in fine weather than in cloudy weather. In the years in which Type I could not conjugate, cool and cloudy weather continued before and during the usual conjugation period.

In the conjugation years, respiratory activity of intact cells increased temporarily at an early stage of the initiation of the conjugation process, thereafter decreased sharply in the later stages of conjugation, and reincreased slightly in the newly formed zygote stage (Fig. 2: Type I, A, B, and C; Type II, A, E, and F). In the nonconjugation years, the activity increased temporarily at the same period as increase of respiration in conjugation years, and thereafter decreased to the original level (Fig. 2: Type I, E and F; Type II, H). Degree of change in respiratory activity was greater in conjugation years than in nonconjugation years. The respiratory activity changed during conjugation process at the usual period for seven years from 1967 to 1972.

The activity of intact cells was not stimulated by addition of glucose or succinate.

Conjugation period and activity of photo-evolution of oxygen: As seen in Table 1, the activity of oxidation of reduced indigocarmine in light decreased during the conjugation process.

Activities of respiratory enzymes in mitochondrial fraction: As seen in Table 2, the activities of NADH-oxidase, cytochrome *c* oxidase, NADH-cytochrome *c* reductase, and succinate-cytochrome *c* reductase in mitochondrial fraction and crude extract decreased during the conjugation process in parallel with that of endogenous respiration of intact cells.

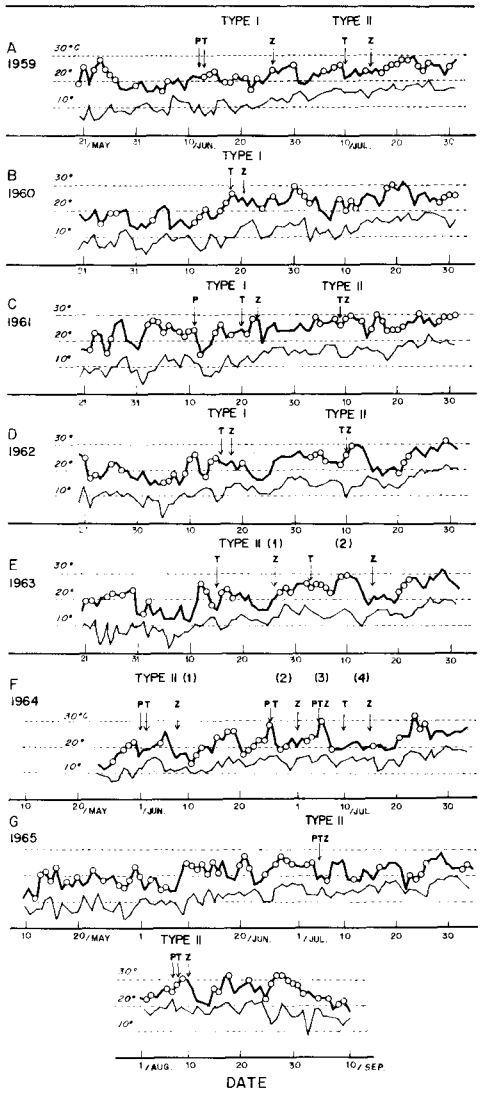

Fig. 1. Conjugation periods of two types of *Spirogyra* for several years. V, P, T and Z, vegetatively growing, pairing, conjugating, and zygote stages, respectively. T-stage is including conjugation-tube forming (T_1), conjugation-tube growing (T_2), conjugation-tube connecting (T_3), and protoplasm-translocating (T_4) stages. ○, fine weather; ▬, maximum temperature; —, minimum temperature.

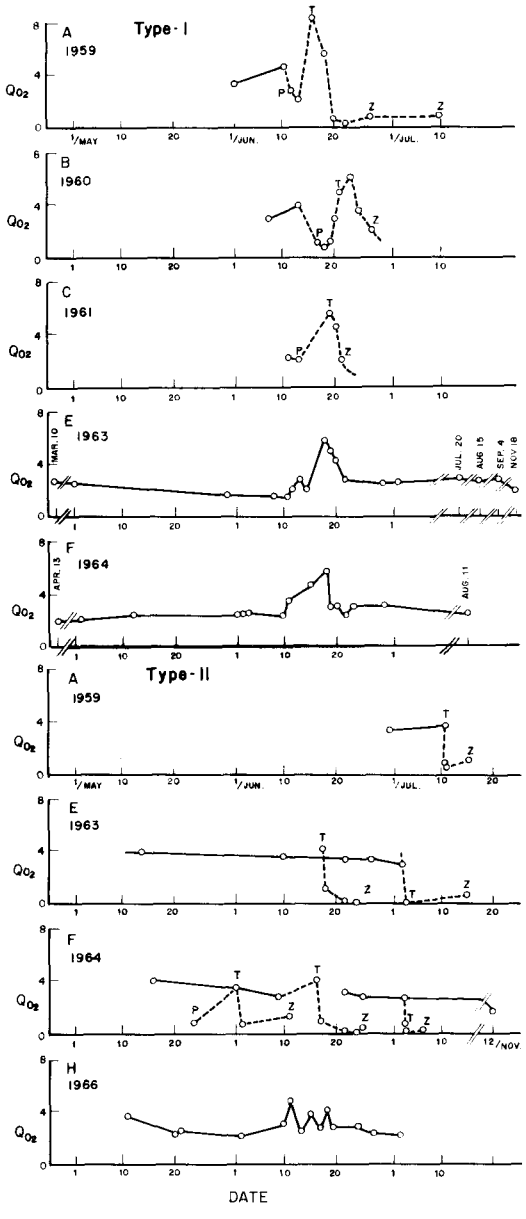

Fig. 2. Relationship between conjugation period and respiratory activity of intact cells. ——, vegetatively growing stage; - - - -, conjugation stage. Type I and Type II were used. (see Fig. 1). Reaction temperature was 30°C except for 25°C in Type I, C.

TABLE 1. Change in activity of photo-oxidation of indigocarmine during conjugation process

Cell Type	Stage	Activity (nmoles, indigocarmin oxidized/mg d. wt./10 min)
I	V	81.7
	T	54.8
	Z	33.0
	V[1)]	20.0
	Z[2)]	22–25
II	V	8.5
	T	6.0
	Z	0.7

1) Cells remained as V-cells at usual conjugation period.
2) Zygote after 11 days of termination of conjugation.

TABLE 2. Changes in activities of NADH-cytochrome *c* reductase, succinate-cytochrome *c* reductase, cytochrome *c* oxidase, and NADH-oxidase in cell-free preparations during conjugation process

Cell Type	Stage	Qo_2	Cell-free prep.	Activity (nmoles/mg protein/min) NADH-cyt. *c* reductase (cyt. *c* reduced)	Succ-cyt. *c* reductase (cyt. *c* reduced)	Cyt. *c* oxidase (cyt. *c* oxidized)	NADH-oxidase (NADH oxidized)
I	V	4.4	Crude ext.	8.5	1.1	0.8	1.0
	P	3.3	»	—	0.9	1.7	1.9
	T_1	4.1	»	5.5	0.5	0.7	2.8
	T_2	1.0	»	0.0	0.3	0.7	1.1
	T_3	0.1	»	0.0	0.5	0.2	0.0
	T_4	0.1	»	0.0	0.0	0.0	0.0
	Z	0.8	»	0.0	0.5	0.0	0.0
	V	4.1	Mit.[1)] frac.	14.5	4.1	1.6	10.0
	T_3	0.1	»	2.7	4.1	0.0	
	Z	0.8	»	0.3	0.0	0.6	0.3
II	V	2.9	»	11.6	3.2	2.6	1.3
	T	0.1	»	2.3	—	1.0	—
	Z	0.9	»	1.1	0.5	0.0	0.0

1) Mitochondrial fraction.

TABLE 3. Changes in activities of photo-reduction of NADP and photo-reduction and photo-oxidation of cytochrome *c* in cell-free preparations during conjugation process

Type	Stage	Cell-free preparation	Activity (nmoles/mg protein/min) NADP reduction (NADP reduced)	Cyt. *c* reduction (cyt. *c* reduced)	Cyt. *c* oxidation (cyt. *c* oxidized)
II	V	Crude extract		1.1	0.9
	T	"		0.3	0.7
	Z	"		0.3	0.1
	V	Chloroplast fraction	14.8	3.4	
	T	"	7.7	0.3	
	Z	"	4.2	0.1	

TABLE 4. Change in activity of triosephosphate dehydrogenase in cell-free extracts during conjugation process

Type	Stage	Cell Qo_2	Cell-free extract	Activity (nmoles/mg protein/min) with NAD	with NADP
II	V	2.8	Crude ext.	81	100
	T	0.1	"	11	11
	Z	0.8	"	62	80
	V	2.8	Soluble frac.	1617	1866
	T	0.1	"	0.0	684
	Z	0.8	"	933	1118

TABLE 5. Changes in chlorophyll contents during conjugation process

Type	Cell Stage	Content (ratio) Chlorophyll a	b
I	V	1.00	1.00
	P	1.20	1.00
	T	0.84	0.77
	Z	0.86	0.73
II	V	1.00	1.00
	P	1.15	1.16
	T_2, T_3	0.93	0.99
	Z	0.71	0.70

Activities of photochemical reactions in chloroplast fraction: As seen in Table 3, the activities of NADP reduction, cytochrome *c* reduction, and cytochrome *c* oxidation in light in chloroplast fraction and crude extract decreased during the conjugation process.

Activity of triosephosphate dehydrogenase in soluble fraction: The activities of both the NAD- and NADP-specific enzymes in soluble fraction decreased significantly during the conjugation process, and increased slightly at the new zygote stage (Table 4).

Contents of chlorophyll a and b in cells: The amounts of chlorophyll *a* and *b* were lesser in conjugating cells and in zygote than in vegetative and conjugation-initiating cells (Table 5).

Discussion

As seen in Fig. 1, Type I conjugated in June from about the 10th to the 20th for several years and Type II around the 10th of July after and during fine weather. In the years in which Type I did not conjugate (*e. g.* 1963, 1964), Type II conjugated several times from the earlier part of June to July. Thus, it seems that the conjugation of Type I is restricted only once a year for a limited period, the 10th to the 20th of June, and that of Type II several times in the period from June to July. In the years in which Type I grew fully, Type II grew in the same pond only after the termination of the conjugation process of Type I and conjugated from July to August. However, in the years in which Type I did not grow normally and did not conjugate, Type II grew from early spring and conjugated several times from June to August after or during fine weather. It seems that in the years in which Type I could not conjugate, cool and cloudy weather continued before and during the usual conjugation period, the 10th to the 20th of June. The length of day-light and fine weather may be important factors for induction of the conjugation process of Type I. The length of day-light in Sapporo during June from the 10th to the 20th is about 15 hours and a quarter. The summer solstice is the 21st or the 22 nd of June, suggesting that the conjugation of *Spirogyra* is related to photoperiodic phenomenon as seen in higher plants, and that length of day-light and fine weather in June are "switch on" factors for the induction of the sexual process.

The activity of endogenous respiration of intact cells temporarily increased prior to initiation of conjugation, thereafter decreased sharply during later stages, and reincreased slightly during new zygote stage (Fig. 2). In nonconjugation years (*e. g.* 1963 and 1964 for Type I, and 1966 for Type

II), the activity temporarily increased during the usual conjugation period (the 10th to the 20th of June for Type I), thereafter decreased to the original level. Degree of the change is greater in conjugation years (Fig, 2: A, B, and C for Type I; A, E, and F for Type II) than in non-conjugation years (Fig. 2; E and F for Type I; H for Type II). These findings suggest that temporal increase of respiratory activity may be required to change metabolic pattern of vegetative growth to that of sexual process, and that specific substance(s) which is produced under fine weather conditions may be required for inhibition of reversion of the change.

It was evident that decreases in respiratory and photochemical activities of intact cells during the conjugation process may be caused by decreases in enzyme contents as shown by experiments with cell-free enzyme preparations (Tables 1 to 4). The findings that amounts of various enzymes decrease during the conjugation process suggest that certain repression factor(s) which act on synthesis of these enzyme proteins may be synthesized in the conjugation stage.

It has been reported that activity of polysaccharide-degradative enzyme increased and electron microscopic structure of cell surface, such as slime and cell wall layers, was destructed during the conjugation of *Spirogyra* (YAMASHITA *et al.*, 1968). We suppose that cell wall-destructive enzyme has a role for fusion of gametes by the softening of cell surface. Cell wall-synthesizing activity increased at the stage of maturation of zygote. These findings suggest that certain substance(s) having a function to activate the synthesis of specific kinds of enzymes, conjugation-specific enzymes, is formed at the conjugation stage.

At the present time, studies on regulatory mechanism of activation and repression of synthesis of specific kinds of enzyme protein in relation to the conjugation phenomenon of *Spirogyra* and *Closterium* are in progress.

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